THE HYDROLYSIS OF N-BENZOYL-L-ARGININAMIDE BY CRYSTALLINE TRYPSIN

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Previous studies of the trypsin-catalyzed hydrolysis of N-benzoyl-L-argininamide to N-benzoyl-L-arginine and ammonia (1, 2) have led to the conclusion that the reaction is first order with respect to both enzyme and substrate and that for any single experiment the rate of hydrolysis may be described by the equation

$$-\frac{ds}{dt} = K'es \tag{I}$$

where e = the total enzyme concentration, s = the substrate concentration, t = the time, and K' = the proteolytic coefficient (3) of the system.

Reinvestigation of the above reaction over a greater range of initial substrate concentrations has now shown that the initial reaction velocity at 25° is essentially independent of the initial substrate concentration for concentrations varying from 0.01 to 0.05 M and that, for these and lesser substrate concentrations, the initial reaction rates obey the Michaelis-Menten (4) equation

$$-\frac{ds}{dt} = \frac{Vs}{K_m + s} \tag{II}$$

within the limits of experimental error. At 25° and pH 7.7 K_m was found to have an apparent value of approximately 0.002 M N-benzoyl-L-argininamide and was shown to be temperature-dependent (Table I). Further it has been observed that for any initial substrate concentration s_0 the reaction rate decreases somewhat more rapidly with time than was expected for a reaction which is truly first order with respect to the substrate concentration and that the reaction products have a marked inhibitory effect upon the reaction rate although ammonium ion alone is without effect.

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Consider the equilibria

$$\underset{(e-p-q)}{\text{E}} + \underset{(s)}{\text{BAA}} \xleftrightarrow{K_m} \underset{(p)}{\text{E·BAA}} \xrightarrow{k} \text{E} + \text{BA} + \text{A}$$

$$\text{E} + \underset{(s_0-s+I)}{\text{BA}} \xleftrightarrow{K_i} \underset{(q)}{\text{E·BA}}$$

where BAA = N-benzoyl-L-argininamide, BA = N-benzoyl-L-arginine, A = ammonia, e = the total enzyme (E) concentration, s = the substrate concentration, s_0 = the initial substrate concentration, p = the concentration of the enzyme-substrate complex, q = the concentration of the enzyme-hydrolysate complex, I = the initial concentration of the hydrolysis products, and $e-p-q$ = the free enzyme concentration.

$$K_m = \frac{s(e-p-q)}{p} \tag{III}$$

$$K_i = \frac{(s_0-s+I)(e-p-q)}{q} \tag{IV}$$

From equation (IV)

$$q = \frac{(s_0-s+I)(e-p)}{K_i+(s_0-s+I)}$$

Upon substitution into equation (III)

$$p = \frac{esK_i}{K_m(K_i+s_0-s+I)+sK_i}$$

If $K_i = rK_m$ (r = the ratio of the inhibitor constant to K_m) and $-ds/dt = kp$,

$$-\frac{ds}{dt} = \frac{kes}{K_m + \dfrac{(s_0+I)}{r} - \dfrac{(1-r)s}{r}} \tag{V}$$

Integration gives

$$ket = 2.3\left[K_m + \frac{1}{r}(s_0+I)\right]\log\frac{s_0}{s} - \frac{(1-r)}{r}(s_0-s) \tag{VI}$$

With $I = 0$,

$$-\frac{ds}{dt} = \frac{kes}{K_m + \dfrac{s_0}{r} - \dfrac{(1-r)s}{r}} \tag{VII}$$

With $t = 0$,

$$-\frac{ds}{dt} = \frac{kes_0}{K_m + s_0} \tag{VIII}$$

With $r = 1$,

$$-\frac{ds}{dt} = \frac{kes}{K_m + s_0 + I} \tag{IX}$$

and the reaction would appear to be exactly first order with respect to substrate.

An equation similar to equation (IX) can be obtained from Klotz's linear equation (5, 6) for the binding of ions by a protein with n equivalent binding sites per molecule; *i.e.*,

$$\frac{e}{A_e} = \frac{K}{n} \cdot \frac{1}{A} + \frac{1}{n} \tag{X}$$

where e = the protein (enzyme) concentration, A = the ion concentration, in this case the concentration of the substrate plus the hydrolysis products, and A_e = the number of moles of ions bound to protein per unit volume of solution.

If one takes the reciprocal,

$$A_e = \frac{enA}{K + A}$$

and assumes that only the substrate combines with the enzyme,

$$s_e = \frac{ens}{K + s}$$

If the rate of reaction is proportional to s_e, the bound substrate concentration,

$$-\frac{ds}{dt} = \frac{Kens}{K + s} \tag{XI}$$

Equation (XI) has the same form as the Michaelis-Menten equation, but with a different significance applied to the constants.

If one assumes that the combining sites of the enzyme may be occupied equally well by either the substrate or the hydrolysis products,

$$s_e = \frac{s}{A} A_e = \frac{s}{A} \cdot \frac{enA}{K + A}$$

and

$$-\frac{ds}{dt} = \frac{kens}{K + A} \tag{XII}$$

If, in equation (VI), r is less than 1, the hydrolysis products must be more firmly bound to the enzyme than is the substrate. In such a case, as the reaction proceeds the rate should decrease more rapidly than would be predicted on the basis of a first order reaction. Such deviations have been observed in all experiments in which the initial substrate concentrations s_0 were relatively high, and at 25° an assumed value for r of 0.5 appeared to fit such cases fairly well. However, with low initial substrate concentrations the deviation was not observed, most likely because of experimental difficulties. The rate constants k, calculated from equation (VIII), are given for various initial substrate concentrations, enzyme concentrations, and temperatures in Table I.

TABLE I

Rate Constants at pH 7.7

k = mM minutes^{-1} (mg. of enzyme nitrogen)$^{-1}$.

s_0*	25°; K_m = 0.0021 M		30°; K_m = 0.0033 M	40°; K_m = 0.0082 M
(1)	e = 0.055† (2)	e = 0.034† (3)	e = 0.034† (4)	e = 0.064† (5)
0.050		0.0022		
0.043			0.0065	0.0074
0.030		0.0024	0.0067	0.0076
0.010	0.0023		0.0060	0.0076
0.0087			0.0066	
0.0058			0.0061	0.0073
0.0050	0.0023			
0.0046		0.0018		
0.0040	0.0020			
0.0030	0.0021	0.0021	0.0066	0.0074
0.0020	0.0023			

* mM of N-benzoyl-L-argininamide per ml.

† Mg. of protein nitrogen per ml.

Experimental values for the extent of hydrolysis ($s_0 - s$) at 25° are compared in Table II with those calculated from equation (VI) with values of r = 0.5, 1, and ∞. In general the agreement is better for r = 0.5, although, as pointed out previously at very low initial substrate concentrations, a better fit is obtained with r = 1.

The effect of the reaction products upon the rate of hydrolysis of N-benzoyl-L-argininamide by trypsin is probably more clearly shown in Table III. It will be seen that 0.05 M ammonium ion causes no demonstrable effect. Because of the low solubility of N-benzoyl-L-arginine it was not possible to determine its effect upon the system directly and it is surprising that it was not precipitated from the reaction mixture. If it

TABLE II

Comparison of Experimental and Calculated Results at 25° and pH 7.7

K_m = 0.0021 M N-benzoyl-L-argininamide; k = 0.0022 mM minutes^{-1} (mg. of enzyme nitrogen)$^{-1}$; e = 0.055 mg. of protein N per ml.

s_0	Time	Hydrolysis	$s_0 - s$, mM per ml. × 10^3			
			Observed	Calculated, r = 0.5	Calculated, r = 1.0	Calculated, $r = \infty$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
mM per ml.	*min.*	*per cent*				
0.010	11.5	11.2	1.12	1.05	1.11	1.13
	20	17.0	1.70	1.73	1.83	1.98
	40	28.5	2.85	2.95	3.31	3.88
0.005	10	16.2	0.81	0.78	0.80	0.83
	20	29.8	1.49	1.35	1.47	1.65
	30	37.0	1.85	1.83	2.02	2.37
	40	45.8	2.29	2.25	2.49	2.97
	50	51.0	2.55	2.57	2.88	
	60	56.8	2.84	2.85	3.20	
0.004	10	16.7	0.67	0.67	0.73	0.78
	20	30.0	1.20	1.22	1.31	1.47
	30	41.8	1.67	1.65	1.79	2.08
	40	48.3	1.93	1.98	2.18	2.55
	50	59.0	2.36	2.26	2.51	
	60	62.8	2.51	2.49	2.77	
0.003	10	20.0	0.60	0.60	0.57	0.67
	20	32.0	0.96	1.05	1.14	1.25
	30	44.7	1.34	1.43	1.53	1.78
	40	59.0	1.77	1.72	1.84	2.16
	50	65.3	1.96	1.92	2.08	
	60	71.0	2.13	2.06	2.27	
0.002	10	26.5	0.53	0.48	0.52	0.57
	20	46.0	0.92	0.83	0.90	1.00
	30	61.5	1.23	1.08	1.18	1.33
	40	69.5	1.39	1.27	1.39	1.57
	50	70.5	1.41	1.42	1.54	1.76
	60	80.5	1.71	1.53	1.68	

had, upon reaching the concentration u, the rate from that time on would be expressed by the equation

$$-\frac{ds}{dt} = \frac{kes}{\left(K_m + \frac{u}{r}\right) + s} \qquad \text{(XIII)}$$

Consequently, the plot of log s (as ordinate) against time would give a curve which would be concave upward from zero time to the time when the concentration of N-benzoyl-L-arginine became equal to u and con-

cave downward from that time to the end of the reaction. Such an effect was not observed in any of the experiments. In order to circumvent the difficulty occasioned by the low solubility of N-benzoyl-L-arginine, a solution 0.05 M in N-benzoyl-L-argininamide was completely hydrolyzed, the enzyme destroyed by heating, and fresh substrate and enzyme added. The rate observed in this solution which was initially 0.042 M in benzoyl-L-arginine and ammonium ion was markedly less than that noted in the absence of added hydrolysis products and agreement between observed and calculated values was good (Table III). While these data offer support for the postulate that the hydrolysis products function in an inhibitory manner, it should be pointed out that Schwert *et al.* found no evi-

TABLE III

Effect of Reaction Products on Rate of Hydrolysis at 25° and pH 7.7

K_m = 0.0021 M N-benzoyl-L-argininamide; k = 0.0022 mM minutes^{-1} (mg. of enzyme nitrogen)$^{-1}$; e = 0.077 mg. of protein nitrogen per ml.; s_0 = 0.050 mM per ml.

Reaction products added (1)	Time (2)	Hydrolysis (3)	$s_0 - s$, mM per ml. × 10^3		
			Observed (4)	Calculated, r = 0.5 (5)	Calculated, r = 1.0 (6)
	min.	*per cent*			
None	60	16.8	8.4	8.3	9.0
	120	29.0	14.5	14.3	16.2
	270	50.0	25.0	25.0	29.3
0.05 M NH_4^+	60	16.9	8.5	8.3*	9.0*
	120	29.4	14.7	14.3*	16.2*
0.042 M N-benzoyl-L-arginine, 0.042 M NH_4^+	60	8.3	4.1	3.7†	5.2†
	120	15.5	7.8	6.7†	9.8†
	240	27.3	13.6	12.5†	17.5†

* Assuming no inhibition by ammonium ion.

† Assuming inhibition by N-benzoyl-L-arginine.

dence of inhibitory action by the hydrolysis products in the trypsin-catalyzed hydrolysis of N-benzoyl-L-arginine methyl ester (7) and it may be that both ammonium ion and N-benzoyl-L-arginine are necessary for inhibitory action.

EXPERIMENTAL

Reagents—The data presented were obtained with a preparation of crystalline trypsin procured from Armour. Similar results were obtained with a Lehn and Fink preparation. Benzoyl-L-argininamide hydrochloride was prepared according to the procedure of Bergmann, Fruton, and Pollok (8). The 0.1 M phosphate buffer (pH 7.9) was prepared from

potassium dihydrogen phosphate and disodium hydrogen phosphate. Reagent grade formaldehyde, 40 per cent by volume, was shaken with basic magnesium carbonate, filtered, and the filtrate (pH 8) used for the formol titrations (9–11). All solutions were prepared with water redistilled from an all-glass apparatus.

Procedure—Generally 5.0 ml. of substrate solution and 1.0 ml. of buffer solution were pipetted into a 1 × 6 inch test-tube, the tube placed in a thermostat, 1.0 ml. of enzyme solution added, the solution shaken, and a 1.0 ml. aliquot immediately withdrawn for a blank determination. After suitable time intervals, additional 1.0 ml. aliquots were withdrawn. Each aliquot was added immediately to an equal volume of formaldehyde solution contained in a 10 ml. beaker, and the beaker placed in the depression of a rubber stopper which was mounted on the shaft of an inverted air-driven stirrer in such a way as to permit rotation of the beaker during the titration. The glass and reference electrodes immersed in the solution facilitated stirring. The solution was titrated with standard 0.01 M sodium hydroxide in a semiautomatic burette graduated in 0.01 ml. and equipped with a capillary tip of sufficient length to permit introduction of the reagent beneath the surface of the solution being titrated; thus the disadvantages inherent in drop-wise transfer are avoided. The end-point of the titration (pH 8.1) was determined with the aid of a Beckman model G pH meter equipped with electrodes specially constructed for use in the limited space available. The glass electrode was of the type intended for use in alkaline solutions. After every titration the electrodes were washed with distilled water and checked against a standard buffer solution. The solutions were invariably titrated immediately after the aliquot had been added to the formaldehyde solution. Blank runs made with solutions containing no enzyme indicated that the substrate was not hydrolyzed in the absence of the enzyme under the conditions employed.

SUMMARY

A reinvestigation of the kinetics of hydrolysis of N-benzoyl-L-argininamide by crystalline trypsin has led to the conclusion that the hydrolysis products enter into the over-all reaction as inhibitors.

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